Featured Perspective

Limb Patterning: From Signaling Gradients to Molecular Oscillations

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Abstract

The developing forelimb is patterned along the proximal–distal and anterior–posterior axes by opposing gradients of retinoic acid and fibroblast growth factors and by graded sonic hedgehog signaling, respectively. However, how coordinated patterning along both axes is accomplished with temporal precision remains unknown. The limb molecular oscillator *hairy2* was recently shown to be a direct readout of the combined signaling activities of retinoic acid, fibroblast growth factor and sonic hedgehog in the limb mesenchyme. Herein, an integrated time-space model is presented to conciliate the progress zone and two-signal models for limb patterning. We propose that the limb clock may allow temporal information to be decoded into positional information when the distance between opposing signaling gradients is no longer sufficient to provide distinct cell fate specification.

***Legend.** The embryo molecular clock (EC), based on hairy2 gene expression, operating in the distal limb mesenchyme has been recently identified to be influenced by the RA, FGF and SHH signaling pathways, which govern the proximal-distal and anterior-posterior limb patterning. Since the EC gene, hairy2 expression lies at the intersection of the key limb signaling pathways, we propose that the notion of time provided by EC oscillations to the distal limb progenitor cells as the fourth dimension of limb development.*

Embryo limb development has been thoroughly scrutinized in search for the molecular mechanisms involved in translating gradients of morphogen activity into positional information over time and

space. The limb distal apical ectodermal ridge (AER) produces fibroblast growth factors (FGFs) that drive proximal–distal (PD) outgrowth and patterning [1] *via* Erk/MAPK and Akt/PI3K pathway activation [2,3]. FGF activity is counteracted in the proximal limb by retinoic acid (RA) produced by the embryo flank [4]. The zone of polarizing activity (ZPA) in the posterior distal limb is a signaling center producing sonic hedgehog (SHH), which mediates anterior–posterior (AP) limb patterning through modulation of Gli1–Gli3 transcription factor activities [5,6]. Different models for limb patterning have been proposed; however, none accounts for coordinated spatiotemporal patterning along both PD and AP axes. Two recent publications studied the dependence of *hairy2* gene expression on the major signaling morphogens operating during limb development—FGF, SHH and RA [7,8]. The results obtained position the *hairy2* molecular oscillator at the intersection of the multiple signaling activities driving limb PD and AP outgrowth and patterning.

Limb PD patterning is currently explained by the two-signal model [9]. Here, the three limb elements (stylopod, zeugopod and autopod) are established by opposing signaling gradients of AER-derived FGFs and RA produced in the embryo flank. Over time, these morphogen sources are gradually distanced in space, generating appropriate thresholds of signaling activity where the limb element boundaries are established. Data accumulated over the past years clearly show the imperative requirement of AER-FGF [10,11] and flank-RA [12,13] signaling, as well as their opposing activities [14,15] for proper limb PD outgrowth and patterning. However, how diffuse morphogen gradients can generate limb element boundaries with such precision remains to be clarified. Importantly, time-dependent responses to these two opposing limb signaling activities have been reported [1,15]; distal limb cells lose their ability to respond to RA over time, even when a local source of RA is provided [15] and limb truncations resulting from AER ablation are less severe when the manipulation is performed later in time [1]. This could be explained as a consequence of irreversible cell fate commitment progressively acquired by the distal limb tissue over time. Time also assumes an important dimension in acquisition of cell identity along the limb AP axis since the duration of tissue exposure to SHH signaling is also a crucial factor in digit specification [16–18].

The existence of a time-counting mechanism operating in the distal limb mesenchymal cells was long proposed by the progress zone model for limb PD patterning [1]. A molecular clock in the developing limb was reported in 2007 by showing that the HES (*h*airy *e*nhancer of *s*plit) *hairy2* transcription factor presented gene expression oscillations in the chick distal limb with a 6-h periodicity [19], corresponding to half the time required to specify a limb skeletal element [19,20]. *hairy2* expression appears in the presumptive forelimb region as early as stage HH14, is uniformly expressed in the entire limb mesenchyme until stage HH17 and displays positive and negative expression domains at stages HH18/HH19. From HH20 to HH28, *hairy2* expression oscillates in the chondrogenic precursor cells along PD and AP axes [19]. A similar molecular clock has long been known to underlie timely axial body segmentation during somitogenesis [21], and an array of other biological systems also possess functional cycles of *HES* gene expression [22]. During somitogenesis, synchronous *HES* gene oscillations function cooperatively with counteracting gradients of FGF and RA activity to translate temporal information into spatial patterns along the AP body axis [23,24]. Resende *et al.* further described a crucial role for notochord-derived SHH signaling in control of somitogenesis clock periodicity [25]. Hence, this well-established molecular clock relies on FGF, RA and SHH morphogens for its functional role in embryo body segmentation. Interestingly, these are precisely the signaling molecules overseeing limb PD and AP outgrowth and patterning, raising the hypothesis that a limb molecular clock could also be playing a role in translating temporal into spatial information in this system.

Addressing this issue, two recent publications have characterized the influence of AER-FGF, ZPA-SHH and flank-RA signaling activities on *hairy2* expression in the chick forelimb bud [7,8]. *hairy2* expression in the undifferentiated distal limb tissue closely reflects the dynamics of the limb signaling centers activities [7]. AER-FGFs were found to be short-term, short-range instructive signals on distal limb *hairy2* expression through p-Erk, while ZPA-SHH acts as a long-term, long-range permissive signal [7]. Gli3, a crucial effector of SHH signaling in the developing limb [5,26], oversees tissue permissiveness for *hairy2* expression in such a way that FGF instructive signaling can only induce *hairy2* expression in the presence of low relative amounts of Gli3 repressor form (Gli3-A/Gli3-R $>$ 1). Consequently, *hairy2* expression pattern in the distal limb mirrors Gli activation status along the AP axis of the tissue: (1) *hairy2* expression is excluded from the anterior-most limb, where Gli-R activity is predominant (Gli-A/Gli-R $<$ 1). Accordingly, expression of the *hairy2* homolog *hes1* gene expands anteriorly in Gli3 conditional knockout mice limbs [27], suggesting that the regulatory role of Gli3 on *HES* expression is a conserved trait across species; (2) ZPA-SHH ensures permanent *hairy2* expression in the posterior distal limb by restricting Gli-R levels (Gli-A/Gli-R $>$ 1); (3) in the medial region of the distal AP axis, an intermediate state of Gli activity is established and *hairy2* expression is now oscillatory in nature. Proximal RA was further shown to positively regulate *hairy2* expression as both instructive and permissive signals by activating p-Erk and relieving Gli-R-mediated inhibition, respectively, while BMP4 inhibited *hairy2* [8]. Combining the effects

observed for FGF, RA and SHH on *hairy2* expression, the emerging overall picture is that the spatial distribution of these morphogen gradients and resulting intracellular signaling activities matches the expression patterns/domains of *hairy2* over time in limb development (Fig. 1). Recent studies have elegantly shown that morphogen gradients are capable of generating distinct patterns of transcription factor gene expression, ranging from stripes to oscillations, and suggest that this mechanism could underlie the somitogenesis molecular clock [28,29]. Considering that *hairy2* off/oscillatory/on states result from graded morphogen activities, it is tempting to postulate that a similar mechanism could also generate *hairy2* oscillations in the limb. The molecular components of a putative gene regulatory network operating in the limb remain to be identified, and our data suggest that Hairy2 could be a candidate. However, further knowledge of Hairy2 action on the limb signaling molecules such as SHH, Gremlin, BMPs and FGFs will be crucial for establishing Hairy2 as a core component of such network. Variations in Erk phosphorylation levels in the distal limb could also underlie oscillations of *hairy2* expression, as they are known to drive *hes* gene oscillations in both mouse PSM [30] and C3H10T1/2 mesenchymal stem cells [31].

We propose that, in the early limb bud (Fig. 1a), RA (permissive and instructive) and FGF8 (instructive) signaling underlie constant *hairy2* expression. BMP signaling is suppressed by RA [32,33] and by Gremlin (GREM) [34], ensuring low levels of Gli-R and constant *hairy2* expression. Over time, the distal limb becomes progressively distanced from the RA source and is protected from RA influence by FGF induced *cyp26* expression [35]. Under these conditions (Fig. 1b), BMP4 in the anterior distal limb is relieved from RA-mediated inhibition [32,33], favoring Gli3-R activity [36] and Gli3-A/Gli3-R < 1, which inhibits *hairy2* expression. Gli3-R induces *bmp4* [37] and excludes *grem* expression in the anterior limb [38–40], further

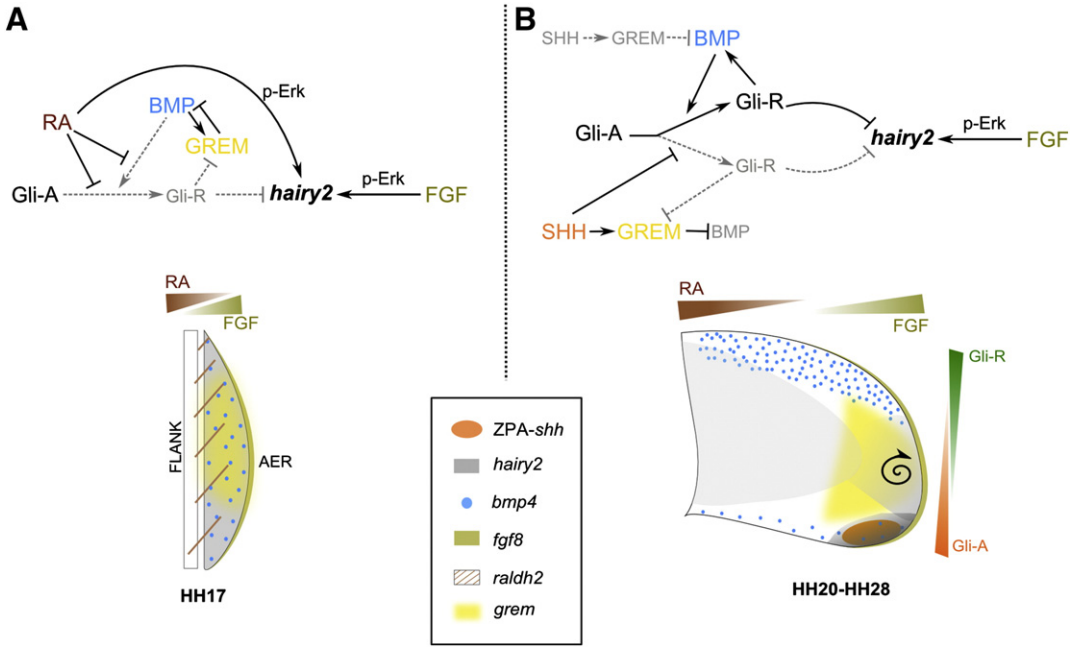

Fig. 1. Integrated time-space model for limb PD/AP patterning: *hairy2* expression during limb development reflects the integrated signaling activities mediating both PD and AP limb patterning. (A) In the early limb bud (HH17), the entire mesenchyme is under the influence of flank-RA and AER-FGF signaling. RA signaling inhibits Gli-R directly and by counteracting BMP activity, establishing a widespread permissive condition (Gli3-A/Gli3-R > 1) for *hairy2* induction by both FGF and RA signaling though p-Erk. BMP signaling is further antagonized by GREM. (B) At later stages of limb development (HH20–HH28), the distal mesenchyme is distanced from the influence of proximal RA. The distal AP axis displays a gradient of Gli-A/Gli-R levels due to polarized SHH signaling from the ZPA. The anterior limb presents high levels of BMP and Gli-R, inhibiting *hairy2* expression. Intermediate levels of Gli-A/Gli-R in the medial distal mesenchyme allow for oscillatory *hairy2* expression. The presence of GREM in that tissue may contribute to appropriate Gli-A/Gli-R levels by counteracting BMP signaling. In the posterior limb, high levels of Gli-A/Gli-R and FGF produce permanent *hairy2* induction. This signaling scenario enables off/oscillatory/on *hairy2* patterns, which may underlie cell fate specification over time. *hairy2* oscillations in the distal mesenchyme take place in the chondrogenic precursor cells [19] along both PD and AP axes. Since the limb clock is simultaneously integrating molecular information along these axes, it could play a crucial role for coordinated PD/AP limb outgrowth and patterning over time. Anterior is up; posterior is down; proximal is left; distal is right.

relieving inhibition of BMP activity, ensuring low levels of Gli3-A/Gli3-R. *shh* expression in the ZPA limits Gli-R levels in the posterior distal limb (Gli-A/Gli-R > 1) and establishes a gradient of Gli signaling along the limb AP axis [6], which dictates tissue responsiveness to instructive AER-FGF signaling on *hairy2*. The Gli-A/Gli-R > 1 permissive condition is further corroborated in the medial distal limb by SHH-induced GREM, which represses BMP activity [34]. Hence, the joint action of permissive ZPA-SHH and instructive AER-FGF now shape *hairy2* expression pattern and create the conditions for transcriptional cycles of *hairy2* in the medial distal limb domain.

The newly identified regulatory actions of FGF/RA/SHH limb morphogens on *hairy2* expression suggest that it is possible to conciliate the two-signal and progress zone models, linking spatial morphogenic patterning with temporal precision. Flank-RA signaling, concomitant with non-oscillatory *hairy2* expression until stage HH19 may specify the proximal-most stylopod early in development [41,42]. Over time, the distal mesenchyme (progress zone) escapes RA influence and is under continuous AER-FGF and ZPA-SHH signaling gradients. Under these conditions, *hairy2* presents oscillatory expression in chondrogenic precursor cells [19], which could constitute a time-counting mechanism progressively providing cell positional information for zeugopod (until stage HH23) and autopod specification [41,42]. Towers *et al.* have suggested that a switch from a gradient-based to a clock-based molecular mechanism could underlie limb PD patterning [43]. The reports herein revised [7,8] are in agreement with this hypothesis and present *hairy2* transcription factor as a crucial molecular component integrating morphogen gradient information along limb PD and AP axes. Moreover, the limb AP off/oscillatory/on states of *hairy2* expression in response to graded SHH morphogen signaling strongly support the model for morphogen interpretation proposed by Balaskas *et al.* [28] and place *hairy2* in the transcriptional network underlying graded Gli activity interpretation in the developing limb.

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Abbreviations used:
RA, retinoic acid; FGF, fibroblast growth factor; SHH, sonic hedgehog; AER, apical ectodermal ridge; PD, proximal–distal; ZPA, zone of polarizing activity; AP, anterior–posterior.

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